REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 10-07-2003

2. REPORT TYPE Final Report

3. DATES COVERED *(From – To)* 01-Jun-02 - 10-Jul-03

4. TITLE AND SUBTITLE

Assessment of the Genotoxic Effects of High Peak-Power Pulsed Electromagnetic Fields

5a. CONTRACT NUMBER ISTC Registration No: 2350

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Nikolai Chemeris

5d. PROJECT NUMBER

5d. TASK NUMBER

5e. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Russian Academy of Sciences
3, Institutskaya Str.
Pushchino 142290
Russia

8. PERFORMING ORGANIZATION REPORT NUMBER

N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

EOARD
PSC 802 BOX 14
FPO 09499-0014

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S) ISTC 01-7011

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This report results from a contract tasking Russian Academy of Sciences as follows: Because of the widespread increase in man-made sources of electromagnetic fields (EMFs), including high peak-power pulsed EMF (HPPP EMF), it has become important to study the health consequences, including possible genotoxic effects of such fields. Because of the simultaneous increase in other environmental toxins, it is difficult to isolate the effects of EMFs in natural human populations. Therefore, this question must be studied under controlled laboratory conditions. The objective of the proposed project is the detection of possible HPPP EMF effects on biological systems at the subcellular and molecular levels. The primary effort will be to assess the effects of HPPP EMF on the genetic material and on intracellular biochemical processes that regulate DNA replication. In doing so, the researchers will develop and modify special methods for the analysis of nucleoid DNA structure on the basis of the single cell gel electrophoresis (comet) assay. Improvements in this method will raise its sensitivity, reliability, and rapidity, as well as decrease its cost. The researchers plan to computerize the method using digital imaging and image analysis techniques. Results will help determine the basic biological effects of HPPP EMF and help provide a broader basis for setting appropriate safety standards.

15. SUBJECT TERMS

EOARD, Biological & Medical Sciences, Radiobiology

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18, NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
UNCLAS	UNCLAS	UNCLAS	UL	28	Valerie E. Martindale
					19b. TELEPHONE NUMBER *(Include area code)* +44 (0)20 7514 4437

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39-18

ISTC 2350

Final
Project Technical Report
of ISTC 2350

Assessment of the Genotoxic Effects of High Peak-Power Pulsed Electromagnetic Fields (EMFs)

(From 1 June 2002 to 31 May 2003 for 12 months)

Nikolai Konstantinovich Chemeris
(Project Manager)
Institute of Cell Biophysics of Russian Academy of Sciences

June 2003

This work was supported financially by European Office of Aerospace Research and Development (EOARD) and performed under the contract to the International Science and Technology Center (ISTC), Moscow.

ISTC 2350

Assessment of the Genotoxic Effects of High Peak-Power Pulsed Electromagnetic Fields (EMFs)
(From 1 June 2002 to 31 May 2003 for 12 months)

Nikolai Konstantinovich Chemeris (Project Manager)
Institute of Cell Biophysics of Russian Academy of Sciences*

The objective of this project is the detection of possible genotoxic effects of high peak-power pulsed electromagnetic fields (HPPP EMF).

The experimental study was performed to assess effects of HPPP EMF on nucleoid DNA structure of living cells *in vitro* (erythrocytes of *Xenopus laevis* frog, human whole blood leukocytes and isolated lymphocytes) with comet assay (single cell gel electrophoresis assay) which is high sensitive method to detect DNA single-strand breaks and alkali-labile lesions. The results obtained allow to conclude that HPPP EMF under the chosen mode of exposure (8.8 GHz, 180 ns pulse width, peak power 65±5 kW, pulse repetition rate of 50 Hz, exposure duration of 40 min) does not cause any specific genotoxic effects on DNA of native frog erythrocytes, human whole blood leukocytes and isolated lymphocytes *in vitro*. The increase in DNA damage after exposure of frog erythrocytes to HPPP EMF was due to temperature rise in the exposed cell suspension

Keywords: High Peak-Power Pulsed Electromagnetic Field, Genotoxic Effects, Comet Assay, DNA damage, erythrocytes of *Xenopus laevis* frog, human whole blood leukocytes, isolated lymphocytes

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*Pushchino, Moscow region, 142290, Russian Federation
Phone +7 (095) 925 59 84, Fax +7 (0967) 790509
E-mail: admin@icb.psn.ru

The work has been performed by
the following institutes.

1. Participated institutes:
    1.1. Leading institute:
Institute of Cell Biophysics of Russian Academy of Sciences (ICB RAS)
Pushchino, Moscow Region, 142290, Russian Federation
Phone +7 (095) 925 59 84, Fax +7 (0967) 790509
E-mail: admin@icb.psn.ru
    1.2. Subcontracted institute:
Russian Federal Nuclear Center - All-Russian Research Institute of Experimental Physics (VNIIEF)
37, Mir Str., Sarov, Novgorod Region, 607190, Russian Federation
Phone +7 (83130) 4 53 73, Fax +7 (83130) 5 38 08

2. Partner
European Office of Aerospace Research and Development (EOARD)
223-231 Old Marylebone Road,
London, NW1 5TH, United Kingdom
Phone +44 207 514 4952, Fax +44 207 514 4960
Email: candy.lndsly@london.af.mil

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1. Introduction

Because of the widespread increase in man-made sources of electromagnetic fields (EMFs), including high peak-power pulsed EMF (HPPP EMF), it has become important to study the health consequences, including possible genotoxic effects of such fields. Because of the simultaneous increase in other environmental toxins, it is difficult to isolate the effects of EMFs in natural human populations. Therefore, this question must be studied under controlled conditions. The objective of the project is the detection of possible HPPP EMF effects on biological systems at the subcellular and molecular levels. The general scope of activities is an assessment of possible genotoxic effects of HPPP EMF in different cell types, including nuclear erythrocytes of amphibians, human blood leukocytes and lymphocytes obtained from fresh donor blood. To achieve the primary goal of the project, the researchers have developed and modified special techniques of single cell gel microelectrophoresis (comet assay) for the analysis of nucleoid DNA structure and detection of primary DNA damage (single-strand breaks and alkali-labile lesions).

The research is a basic research. As a result of these experimental investigations, we can assess possible changes in nucleoid DNA structure or DNA damage caused by the exposure of cells to HPPP EMF. In order to validate our system, the results obtained from HPPP EMF and sham exposure were compared with results from exposure to known genotoxic agents (e.g., ionizing radiation, alkylating agents, etc.). If the results of these experiments indicate a genotoxic effect of HPPP EMF, they will be the basis for potential future research on the mechanisms of the effects as well as providing an input to people concerned about appropriate safety standards for EMF.

The researchers conducted experiments on HPPP EMF effects on the nucleoid DNA structure of living cells *in vitro* (nuclear erythrocytes of amphibians, human blood leukocytes and lymphocytes obtained from fresh donor blood (The fresh blood was purchased from a blood transfusion station that makes it available to any researchers and clinicians on a commercial basis; so there are no human subjects in this study). The basic experimental method is the comet assay, which permits the detection of DNA damage and changes in nucleoid DNA structures [1, 2]. The methodology is based on usage of single cell gel microelectrophoresis, with the damaged DNA migrating to the anode. After a luminescent stain intercalation into DNA, the electrophoresis pattern simulates the formation of "comets" with a bright fluorescent "head" (unimpaired DNA) and fuzzy "tail", the size and fluorescence intensity of which determines the amount of damaged DNA. Currently, the comet assay is used to detect DNA damage in different cells for oncology and autoimmune disease diagnosis, to estimate patient state after treatment with different drugs, and to study mechanisms of DNA damage and repair *in vitro* and *in vivo* [1, 2].

The analysis of DNA comets was performed with the use of unique equipment that is available in researchers' disposal (Laboratory of Regulation in Biomedical Systems of the Institute of Cell Biophysics RAS). The setup includes a modified light microscope with a luminescent header and photometric camera, necessary devices for preparation of gel slides, cell lysis, and microelectrophoresis. The setup is connected to the computer for manipulation, data collection, data storage and data analysis. During the course of the project, we introduced essential modifications into underlying hardware and software to optimize the utility of this instrument for the assessment of HPPP EMF effects, and to enhance reliability, rapidity, and sensitivity of the methods. Because of the high prices of equipment for the exposure of biological samples to HPPP EMF, all HPPP EMF exposures and parallel controls were conducted under subcontract at the Russian Federal Nuclear Center - All-Russian Research Institute of Experimental Physics (Sarov, Novgorod Region), possessing the appropriate equipment.

2. Materials and methods

2.1. HPPP EMF Dosimetry

High-power square microwave pulses (8.8 GHz, 180 ns width, peak power up to 65±5 kW, repetition rate up to 50 Hz) were produced by a "Rodon" transmitter, made on the basis of a pulsed MI-522 magnetron. The pulse width and frequency were determined by settings of the transmitter. Incident power was measured by a wattmeter in composition of a Ya2M-66 measurement block and calorimetric converter of absorbed power made by NNIPI (Nizhniy Novgorod, Russia). The transmitter was set to external triggering from a GI-1 six-channel generator that controlled the repetition rate of pulses. The shape of high-voltage modulating pulses for magnetron supply was monitored on Hewlett Packard 54542A digital oscilloscope (Fig.1).

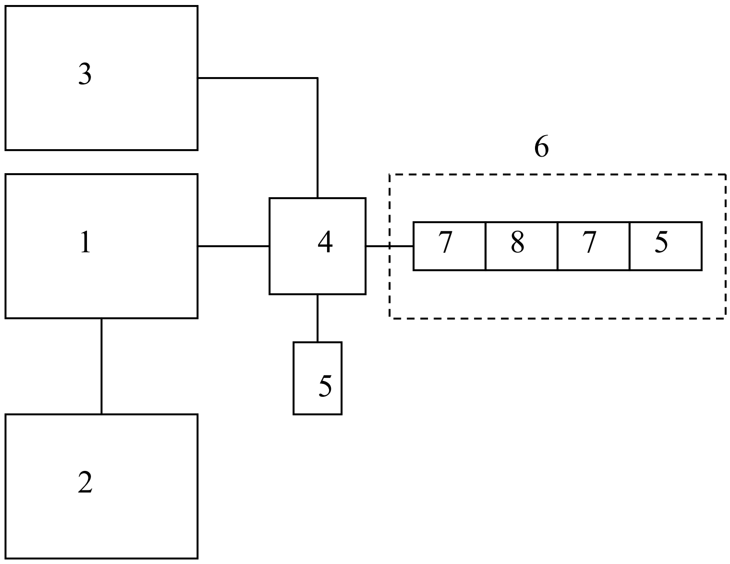

Fig.1. Block-scheme of the experimental set-up on the basis of "Rodon" transmitter. 1 - "Rodon" transmitter; 2 - Digital oscilloscope; 3 - Panorama measurement device; 4 - Waveguide switch; 5 - Matched load; 6 - Experimental line; 7 - Smooth waveguide passage from 23x10 mm^2 to 23x3.4 mm^2; 8 - Waveguide segment with experimental cuvette.

Cell suspension was exposed in special plastic cuvettes of cylindrical shape with a diameter of 10 mm and height of 1 mm put inside a waveguide with a section of 23×3.4 $мм^2$ at a distance of 25-30 mm from waveguide flange. Using the copper-constantan microthermocouple with a diameter of 50 µm, measurements of temperature changes in the experimental cuvette filled with different volumes of a physiological saline (from 10 to 100 µl) were carried out under the exposure to HPPP EMF at an average incident power of 400±10 mW. The thermocouple was introduced into the cuvette bottom through the narrow slot in the large wall of the waveguide (Fig.2). An Agilent Technologies 34970A digital voltmeter was used for the measurement of voltage generated by the thermocouple.

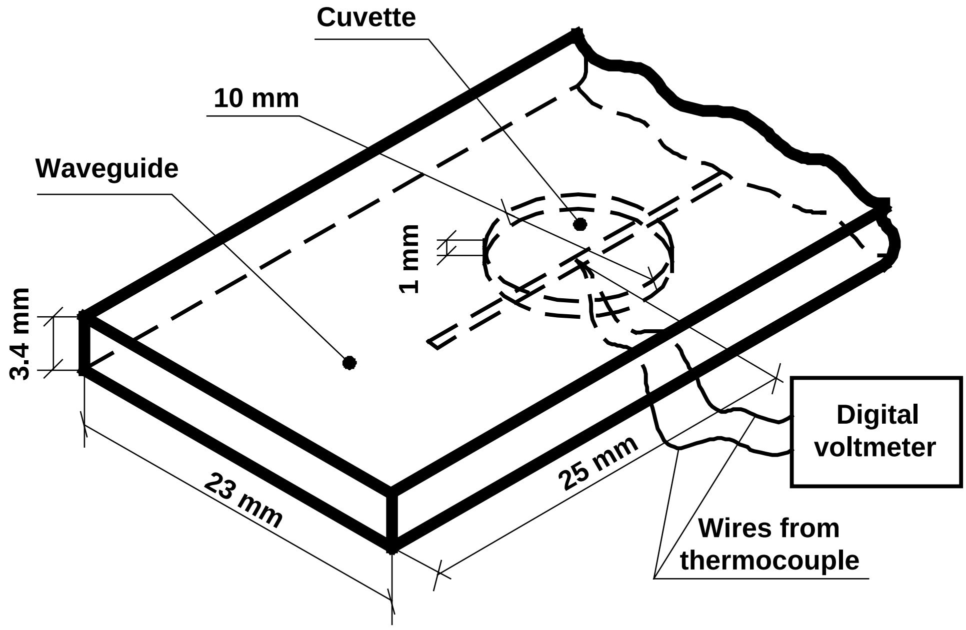

Fig.2. Scheme of temperature measurements in the experimental cuvette exposed to HPPP EMF in rectangular waveguide. The same conditions but without thermocouple were used for blood samples exposure to HPPP EMF.

Obtained temperature kinetics were described as a function

$$\Delta T(t) = c_1[1 - e^{-t/\tau_1}] + c_2[1 - e^{-t/\tau_2}],$$

where c_1 and c_2 are preexponential coefficients, and τ_1 and τ_2 are time constants, from which the level of stationary overheating was determined as

$$\Delta T(\infty) = c_1 + c_2,$$

and the initial rate of temperature rise was determined as

$$\left.\frac{dT(t)}{dt}\right|_{t\to 0} = c_1/\tau_1 + c_2/\tau_2,$$

and corresponding SAR

$$SAR = C\left.\frac{dT(t)}{dt}\right|_{t\to 0},$$

where C is the heat capacity equal to 4200 J/(kg K) for the physiological saline.
For the experimental cuvette filled with 50 µl of physiological saline, from the temperature kinetics it was determined that an average initial rate of temperature rise was about 0.37 ± 0.01^{o}C/s, corresponding SAR was about 1.6 kW/kg (peak SAR was about 300 MW/kg).

For the experimental cuvettes filled with different volumes of physiological saline, temperature kinetics were approximated on the basis of numerical solution of thermal conduction equation

$$\frac{\partial T}{\partial t} = \frac{k}{C\rho} \cdot \frac{\partial^2 T}{\partial x^2},$$

where k is the heat conduction coefficient, ρ is the saline density,
with initial condition

$$\Delta T = \tau_{imp} \cdot SAR/C$$

(sudden changes in temperature ΔT are repeated with a frequency of 50 Hz),

and boundary conditions: a) cuvette bottom is thermally isolated, and b) on the boundary liquid-air

$$\partial Q / \partial t = h \cdot \Delta T_{liq-air},$$

where h is the heat transfer coefficient.
The temperature kinetics obtained from the model calculations are depicted in Fig.3.

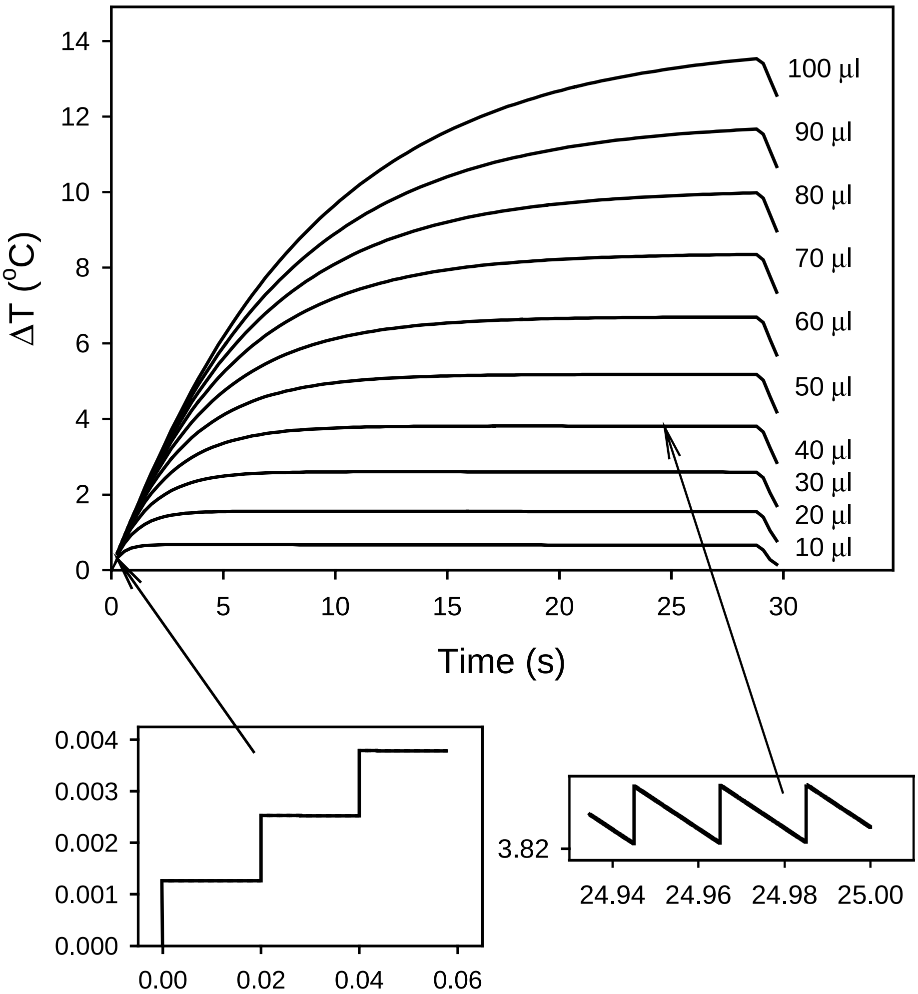

Fig.3. Model temperature kinetics in the experimental cuvette filled with different volumes of physiological saline exposed to HPPP EMF at 200 ns pulse width and pulse repetition rate of 50 Hz.

From the data presented in Fig.3, it is visible that the level of stationary overheating depends on a saline volume. The dependence is depicted in Fig.4.

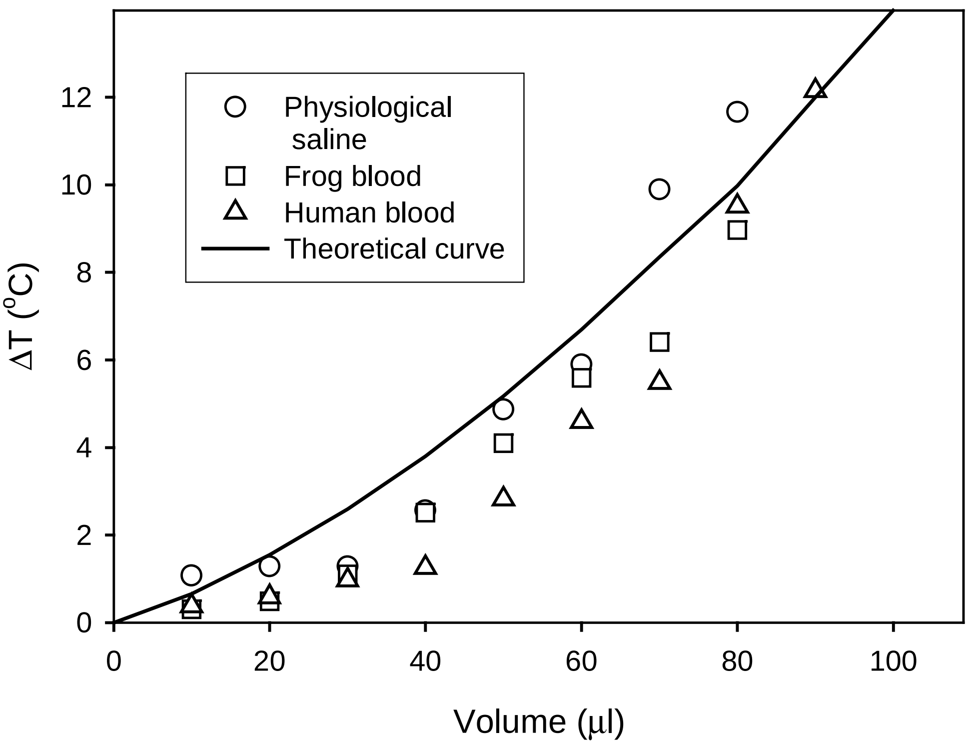

Fig.4. Dependence of the level of stationary overheating of physiological saline, frog blood and human blood on the volume of sample exposed to HPPP EMF. Theoretical curve was calculated for different volumes of physiological saline.

For the volume of 50 μl of frog blood and human blood samples commonly used for the exposure to HPPP EMF, the level of stationary overheating varied in different experiments from 3 to 4.5°C and on the average was about 3.5±0.1°C.

The model calculations of electric field intensity distribution in rectangular waveguide on the H_{10} mode (at an incident power of 70 kW and frequency of 8.8 GHz) were carried out outside of the experimental cuvette and in the cuvette. Maximal electric field intensity in the waveguide with a section of 23x3.4 mm^2 made about 1.42 MV/m, that was approximately two-fold lesser than breakdown electric field intensity for dry air. Maximal electric field intensity in the experimental cuvette filled with a physiological saline with dielectric constant of 59 and loss tangent of 0.46 made about 70 kV/m.

With the help of a waveguide switch, the experimental line was connected to a R2-61 panorama measurement device to determine standing-wave ratio (SWR) and attenuation (A). Measurements of standing-wave ratio and attenuation in a waveguide without an experimental cuvette and with an experimental cuvette (empty and filled with physiological saline) were performed. Considering an average incident power (P_o) of 400 mW and according to the data obtained, the SAR in the experimental cuvette was calculated by the formulas:

$$P_{absorbed} = P_0 - P_{reflected} - P_{transmitted} = P_0\left(1 - \frac{(1-SWR)^2}{(1+SWR)^2} - \frac{1}{10^{A/10}}\right),$$

$$SAR = \frac{P_{absorbed}}{V\rho},$$

where $P_{absorbed}$ is the power absorbed by the saline in the cuvette, V is the saline volume, and ρ is the saline density. The calculations were made on the basis of data on SWR and A obtained at different distances between a waveguide flange and center of the cuvette. The results are shown in Table 1. SAR values obtained from calculations on the basis of both waveguide measurements and recordings of temperature kinetics are in good mutual correspondence.

Table 1. Dependence of SAR on a distance between a waveguide flange and center of the experimental cuvette filled with physiological saline.

Distance from waveguide flange (mm)	SAR (kW/kg)
5	2.1
25	1.9
45	1.8
65	1.6

2.2. Blood Samples and Isolation of Lymphocytes

Male Xenopus laevis, South African clawed toads, were maintained in opaque white plastic tanks at 20-23°C in water from domestic supply dechlorinated by standing for at least 24 h. Frogs were decapitated using scissors and blood was collected in the eppendorf tube with 0.3 ml of cold phosphate-buffered solution (PBS, in mM: 136.7 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4; pH=7.0) with 1 mM EDTA. All animals were treated individually. The vast majority of the cells were erythrocytes because they are much more numerous than any other cell types in the circulatory system. Very few free cells from any other tissues were present. Suspension of erythrocytes was then deluted for a concentration of 1×10^6 cells/ml in PBS with 1 mM EDTA, so that three or four cells would be seen in a single field of 100^x magnification.

The fresh blood from male healthy donors, 25-30 years of age, was purchased from a blood transfusion station that makes it available to any researchers and clinicians on a commercial basis; so there are no human subjects in this study. Sodium citrate was used as an anticoagulant. Whole blood samples were diluted 1:6 with PBS containing 1 mM EDTA, so that final concentration of leukocytes was $1.0\text{-}1.5\times10^6$ cells/ml.

Lymphocytes were isolated by centrifugation of 1 ml of diluted citrate blood in Ficoll-verografin gradient at 600 g using a MiniSpin micro-centrifuge (Germany) [3]. Lymphocyte interphase was collected and washed once in PBS by centrifugation. Sediment was resuspended in PBS containing 1 mM EDTA and diluted to achieve lymphocyte concentration of about 1.5×10^6 cells/ml.

2.3. HPPP EMF Exposure

Blood samples from 15 frogs were used in these experiments. A suspension of erythrocytes prepared for the exposure was divided into three parts, the first of which was used for the negative control (a sample was placed into a not-energized section of the waveguide at a temperature of 25±1°C), the second one was used for sham-exposure (a sample was placed into a not-energized section of waveguide at a temperature of 29±1°C, which imitated the temperature rise induced by the exposure to HPPP EMF), and the third one was used for the exposure. Immediately after these exposures (negative control, sham- or HPPP EMF exposure), a 40-µl aliquot of each part of suspension was used for preparation of microscope slides.

Diluted whole blood samples from 18 donors and suspensions of lymphocytes isolated from blood of 10 donors were used in these experiments. Cell suspension prepared for the exposure was divided into parts, the first of which was used for the negative control (a sample was placed into a not-energized section of the waveguide at a temperature of 23±1°C), the second one was used for sham-exposure (a sample was placed into a not-energized section of waveguide at a temperature of 27±1°C, which imitated the temperature rise induced by the exposure to HPPP EMF), the third one was used for the exposure, the fourth one was used for the second negative control (a sample was maintained in an incubator at 37±0.5°C), and the last one was used for the positive control (cells were incubated at 37±0.5°C in the presence of ethyl methanesulfonate at concentration of 5 mM). Immediately after these exposures (negative and positive controls, sham- or HPPP EMF exposure), a 30-µl aliquot of each part of suspension was diluted 1:1 in PBS containing 1 mM EDTA and used for preparation of microscope slides.
In separate series of experiments, after termination of the exposure, a 30-µl aliquot of each blood sample was diluted 1:1 in PBS (prewarmed to 37°C) containing 1 mM EDTA and then placed in an incubator at 37±0.5°C for 30 min. It would allow detection of any DNA damage expressed after the exposure. Microscope slides were prepared at the end of 30 min of incubation.
In separate series of experiments, after termination of the exposure, a 30-µl aliquot of each blood sample was diluted 1:1 in PBS (prewarmed to 37°C) containing 1 mM EDTA and sodium azide in final concentration of 1 mM and then placed in an incubator at 37±0.5°C for 30 min. This was done to detect any DNA damage which would be repaired by ATP-dependent repair systems during subsequent manipulations with the cells. Sodium azide inhibits cellular ATPase [4 ,5] that uncouples phosphorylation and suppresses ATP-dependent repair processes. Microscope slides were prepared at the end of 30 min of incubation.

The experiments were conducted according to double blind protocols, with application of a randomization at selection of samples for the negative controls, positive control, sham exposure control, and for exposure to HPPP EMF.

2.4. Ionizing Radiation Exposure

The experiments were conducted with cells imbedded into low-melting agarose, i.e. with ready microscope slides kept in ice. The frog erythrocytes were exposed to 0, 50, 100 and 200 cGy of ionizing radiation from a ^{60}Co γ-ray source (Gamma Set-up of Large Energy, Russia, dose rate of 40 cGy/min). The human blood leukocytes and lymphocytes were exposed to 0, 150, 274 and 548 cGy of ionizing radiation from a X-ray source (200 kV, 20 mA, dose rate of 115 cGy/min).

2.5. Treatment with alkylating agent

Blood samples from some frogs used for the experiments with HPPP EMF exposure were treated for 40 min with alkylating agent ethylmethane sulfonate (EMS, 5 mM) at 20±1°C. Human blood samples and isolated lymphocytes were treated for 20 min with different concentrations of EMS (from 0.001 to 10 mM) at 37±0.5°C. EMS was tested, firstly, to check up the method sensitivity, and, secondly, to demonstrate the adequacy of our experimental conditions to detect a known genotoxic agent [2].

2.6. Temperature treatment

Considering the temperature rise under the HPPP EMF exposure, in separate series of experiments we tested the influence of different temperature conditions on occurrence of DNA damage in the cells. Suspension of frog erythrocytes was divided into 3 parts (100 µl each) in PCR 0.25-ml microtubes and incubated simultaneously in a "Tercik" thermocycle (DNA-

Technology, Russia) for 40 min at 20, 25 and 30^{o}C with temperature maintaining accuracy of ±0.5^{o}C. Human blood samples or lymphocyte suspension were divided into 4 parts in PCR microtubes (100 µl) and incubated simultaneously in a "Tercik" thermocycle (DNA-Technology, Russia) for 40 min at 23, 27, 33 and 37^{o}C with temperature maintaining accuracy of ±0.5^{o}C. Then the comet assay was performed with three slide-processing schedules.

2.7. Alkaline Comet Assay (Assessment of Single-Strand Breaks and Alkali-Labile Lesions).

Alkaline comet assay in our modification was used to assess DNA damage in cells (frog erythrocytes, human blood leukocytes and lymphocytes). The comet assay was performed with samples immediately following various exposure conditions. The method was essentially as described in [6, 7] with some modifications. To prepare three-layer microscope slides, a 40-µl aliquot of cell suspension (frog or human blood diluted in PBS with 1 mM EDTA, or lymphocyte suspension) was mixed with equal volume of 1% low-melting-point agarose (Serva) prepared in PBS with 1 mM EDTA and kept at 40±1^{o}C. Fifteen microliters of the mixture was spread (under a cover clip) on each microscope slide prelayered with 0.5% low-melting-point agarose. After solidification of low-melting-point agarose with cells for 5 min at 4^{o}C, a top layer of 15 µl of low-melting-point agarose was added. After the top layer of agarose had solidified for 5 min at 4^{o}C, all slides were immersed in lysing solution and left at 22°C for 25 min in dark. Lysing solution was composed of 2.5 M NaCl, 10 mM Tris-HCl, 1% Triton X-100 (for human leukocytes and lymphocytes, 1% N-lauryl-sarcosine was added), 100 mM EDTA; pH=10.0. Then slides were exposed to an alkaline solution (0.3 M NaOH, 1 mM EDTA; pH=13.0) at 4°C for 20 min in dark. Electrophoresis was performed in the fresh portion of alkaline solution at 4°C for 20 min at 1.9 V/cm for frog erythrocytes and 1.3 V/cm for human blood leukocytes and lymphocytes. The slides were then washed in distilled water for 5 min twice and stained with ethidium bromide (1 µg/ml in PBS) at 22°C for 1 h in dark. Then all slides were coded, each slide was washed in distilled water for 5 min and coverslipped before analysis. All procedures were conducted under yellow light with minimal handling of samples to prevent the occurrence of additional DNA damage in the cells.
Modifications of the equipment used for registration and analysis of images allowed us to establish a mobile variant of photometry setup with total weight of about 3 kg. The slides were examined under a modified "Biolam" microscope (LOMO, Russia) with luminescent header, using a super bright LED with emission maximum of 505 nm for fluorescence excitation (excitation filter 525 nm and barrier filter 590 nm). For recording the images of nucleoids, a LCL-902HS CCD camera with a high sensitivity (0.001 Lux at AGC off) and resolution of 768×494 pixels was used. The software created by us for recording and online image analysis has permitted to achieve a rate of image analysis up to 400 comets per hour. Algorithms for image filtering and smoothing, for calculation of standard parameters of "comets" [8] (amount and percent of DNA in the comet head and tail, radius of the comet head, tail length, tail moment, Olive tail moment, and integral fluorescence intensity in the comet head by a fixed threshold) were realized. Modification of the equipment and software, application of digital methods for registration and image analysis have allowed us to optimize essentially the method, to increase the rapidity and to decrease the cost price of the analysis.

2.8. Data collection, processing and statistical analysis

For each sample (negative control, sham- or HPPP EMF-exposed, ionizing radiation-, temperature-exposed or treated with alkylating agent) photometry was made on 3-5 slides. Routinely, images of 25 cells per slide were scored and processed. Then values of comet parameters were averaged for these 25 cells, mean values and SEMs for each subject (frog or human) were calculated from 3-5 slides, mean values and SEMs for each series were calculated

using mean values for separate subjects (independent experiments). Statistical analysis of the data was based on the Student's *t* test.

3. Results

3.1. Assessment of DNA Damage in Frog Erythrocytes after In Vitro Exposure to High Peak-Power Pulsed Electromagnetic Field

It is known that frogs are resistant for ionizing radiation. This can be determined by both high efficacy of protective systems and genome size of *X.laevis*. In the literature there are no data on assessment of damaging action of ionizing radiation and EMS in erythrocytes of *X.laevis* carried out with the use of the comet assay. Therefore in a special series of experiments, using the comet assay, we assessed sensitivity of isolated erythrocytes of *X. laevis* to action of different doses of γ-ray ionizing radiation (0, 50, 100 and 200 cGy) and directly influencing on DNA alkylating agent EMS in concentration of 5 mM. All standard parameters of comets (amount and percent of DNA in the comet head and tail, radius of the comet head and tail length, tail moment, Olive tail moment, and integral fluorescence intensity in the comet head by a fixed threshold) and variability of these parameters for different animals were analysed. Such extended analysis has shown that the demonstrative quality and quantitative representation of DNA damage in the cells and least variability are given by averaged data on percent of DNA in the comet tail (Table 2). From data shown in Table 2, it is visible that significant difference from corresponding control (0 cGy) for series of comet parameters is displayed already at a dose of 50 cGy. This is clear visible from data on percent of DNA in the comet tail. The percent of DNA in the comet tail at the action of different doses of γ-radiation and alkylating agent is submitted in Table 3.

Table 2. Standard parameters of comets obtained from image analysis of comets of frog erythrocytes exposed to different doses of γ-ray ionizing radiation.

Dose of ionizing radiation (cGy)	Head DNA (arbitrary units)	Tail DNA (arbitrary units)	Head DNA (%)	Tail DNA (%)	Head radius (μm)	Tail length (μm)	Tail moment (arbitrary units)	Olive tail moment (arbitrary units)	Integral intensity by 0.5 level (arbitrary units)
0	190±10	2.7±0.8	98.5±0.5	1.5±0.5	35.3±0.7	10.1±3.1	0.6±0.2	0.8±0.3	153±8
50	170±7	8.4±2.5	95.1±1.5*	4.9±1.5*	36.6±0.6	29.0±8.0*	3.3±1.3	3.0±1.1	136±6
100	172±6	11.1±3.1**	93.7±1.7**	6.3±1.7**	36.6±0.6	38.4±9.1**	4.2±1.7	3.9±1.3*	139±6
200	163±4**	18.3±3.1***	89.8±1.5***	10.2±1.5***	35.6±0.4	55.9±7.4***	7.5±1.6**	6.5±1.2***	133±4*

Notes. The values are mean (± SEM) calculated from 6 independent experiments. * - *p*<0.05, ** - *p*<0.03, *** - *p*<0.001. P values indicate difference from corresponding control (0 cGy).

Table 3. Percent of DNA in the comet tail in frog erythrocytes exposed to different doses of γ-radiation and treated with 5 mM EMS.

Exposure	Dose or concentration	Tail DNA ± SEM (%)	P (and/or N)
Ionizing radiation	0 cGy	1.5 ± 0.5	-- (6)
	50 cGy	4.9 ± 1.5	p<0.05 (6)
	100 cGy	6.3 ± 1.7	p<0.02 (6)
	200 cGy	10.2 ± 1.5	p<0.0003 (6)
EMS	0 mM	1.2 ± 0.2	-- (8)
	5 mM	4.6 ± 0.9	p<0.004 (8)

Notes. The cells were subjected to comet assay immediately after exposure to ionizing radiation or alkylating agent. The values are average (±SEM), P values indicate difference between exposed and corresponding control samples.

The minimal dose of 50 cGy used by us significantly increased the DNA content in the comet tail by more than 3 times as compared to the corresponding control (0 cGy). EMS in concentration of 5 mM during 40-min incubation at 20^{o}C caused DNA damage comparable with that induced by the dose of ionizing radiation of 50 cGy. The DNA content in the comet tail increased approximately by 4 times as compared to the corresponding control (0 mM). The data obtained show that in spite of highly effective system of DNA repair in frog, the comet assay detects significant DNA damage in frog erythrocytes in vitro exposed to ionizing radiation and 5 mM EMS.

As it is known, intensive EMFs can result in overheating of the object which has appeared in a zone of their action. According to existing data, the heating induces formation of reactive oxygen species in solutions, which interacting with DNA can result in formation of 8-oxoguanine, a biomarker of DNA damage [9], DNA strain breaks [10], hydrolysis of glycosyl bonds [11] and deamination of cytosine [12]. Therefore in a separate series of experiments, we studied the influence of different temperatures of incubation of cell suspension on DNA integrity in erythrocytes. The results indicate the increase in DNA damage in erythrocytes with temperature elevation. The percent of DNA in the comet tail was 0.39±0.13, 0.60±0.12 and 1.23±0.16 after incubation at 20, 25 and 30^{o}C, respectively. The DNA damage caused by incubation of cell suspension at 30^{o}C for 40 min was significantly ($p<0.003$ and $p<0.02$) higher as compared to 20^{o}C and 25^{o}C temperature conditions, respectively.

Thus, the level of DNA damage in frog erythrocytes has expressed dependence on temperature conditions, at which the cell suspension was incubated (Table 4). Considering the DNA damage due to temperature elevation during 40-min exposure to HPPP EMF, we collated the data on incubation of cell suspension at 30^{o}C, sham- and HPPP EMF exposure. For comparison, data on the same temperature conditions, sham- and HPPP EMF exposure were averaged and are submitted in Table 4.

Table 4. Percent of DNA in the comet tail caused by temperature-, sham- and HPPP EMF exposure of frog erythrocytes for 40 min.

Exposure conditions	Tail DNA ± SEM (%)	N	P (compared to 20°C)	P (compared to 25°C)	P (compared to 30°C)
20°C	0.83 ± 0.16	15	--	0.284	0.013
25°C	1.16 ± 0.24	18	0.284	--	0.043
30°C and sham-exposure	2.35 ± 0.53	16	0.013	0.043	--
HPPP EMF-exposure	2.36 ± 0.53	15	<0.01	0.038	0.992

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

Results presented in Table 4 demonstrate the same percent of DNA in the comet tail in sham-exposed and HPPP EMF exposed cells ($P>0.99$). At the same time, these data are statistically different from temperature-exposure at 25°C ($p<0.05$) and 20°C ($p<0.02$).

Thus, on the basis of our results, we conclude that HPPP EMF under the chosen mode of exposure (8.8 GHz, 180 ns pulse width, peak power 65±5 kW, repetition rate 50 Hz, exposure duration of 40 min) does not cause any specific genotoxic effects on DNA of frog erythrocytes *in vitro*. The increase in DNA damage after exposure of cells to HPPP EMF shown in Table 4 is due to temperature rise in the cell suspension by 3.5±0.1°C, this was confirmed in sham-exposure experiments and experiments with incubation of cells for 40 min under corresponding temperature conditions. At such overheating, the effect can be associated with an enhanced rate apurinization/apyrimidinization of DNA at physiological conditions.

3.2. Assessment of DNA Damage in Human Blood Leukocytes and Lymphocytes after In Vitro Exposure to High Peak-Power Pulsed Electromagnetic Field

In connection with the produced modifications of the comet assay, it was necessary to test the sensitivity of the method performed with human whole blood leukocytes and isolated lymphocytes to the action of known damaging factors - ionizing radiation and alkylating agent. With this purpose, a special series of experiments was conducted to investigate the action of X-ray ionizing radiation at doses of 0, 150, 274, and 548 cGy on whole blood leukocytes and isolated lymphocytes. It was shown that occurrence of DNA damage in the cells depends pronouncedly on the dose of ionizing radiation (Table 5).

Table 5. Percent of DNA in the comet tail after action of different X-ray doses on human whole blood leukocytes and isolated lymphocytes.

Dose (cGy)	Whole blood leukocytes		Isolated lymphocytes	
	Tail DNA ± SEM (%)	P (and/or N)	Tail DNA ± SEM (%)	P (and/or N)
0	1.31 ± 0.21	-- (10)	1.03 ± 0.16	-- (7)
150	2.29 ± 0.72	0.102 (4)	3.25 ± 1.21*	0.02 (3)
274	3.49 ± 0.50*	4×10^{-4} (4)	6.22 ± 1.46*	5×10^{-4} (3)
548	8.98 ± 0.73*	10^{-8} (8)	11.41 ± 2.75*	0.002 (6)

Notes. The values are average from independent experiments (±SEM), P values indicate difference between exposed and corresponding control samples (0 cGy). * - significantly different.

From the data submitted, it is visible that isolated lymphocytes of human blood as compared with whole blood leukocytes are more sensitive to the action of X-ray ionizing radiation for all doses used. For example, the X-ray radiation at a dose of 274 cGy increases the DNA content in the comet tail in leukocytes by 2.7 times (p<0.001) and in isolated lymphocytes by more than 6 times (p<0.001).

In a special series of experiments, we studied genotoxic effects of alkylating agent EMS that is advisable for the use as the positive control [2] on human whole blood leukocytes and isolated lymphocytes. Human blood samples and isolated lymphocytes were treated for 20 min with different concentrations of EMS (from 0.001 to 10 mM) at 37±0.5^{o}C.

Table 6. Percent of DNA in the comet tail after action of different concentrations of EMS on human whole blood leukocytes and isolated lymphocytes.

Concentration of EMS (mM)	Whole blood leukocytes		Isolated lymphocytes	
	Tail DNA ± SEM (%)	P (and/or N)	Tail DNA ± SEM (%)	P (and/or N)
0	1.12 ± 0.19	-- (8)	0.97 ± 0.48	-- (5)
0.001	1.51 ± 0.54	0.406 (3)	1.59 ± 0.57	0.450 (3)
0.01	2.43 ± 0.47*	0.012 (3)	1.31 ± 0.48	0.661 (3)
0.1	2.16 ± 0.70	0.071 (3)	1.87 ± 0.68	0.311 (3)
1.0	3.37 ± 0.52*	0.0012 (8)	2.67 ± 0.58	0.054 (5)
2.0	3.79 ± 1.75*	0.028 (3)	--	--
5.0	9.01 ± 2.72*	0.0032 (5)	15.69 ± 9.01*	0.029 (2)
10.0	16.20 ± 3.68*	0.00025 (5)	18.81 ± 5.34*	0.010 (5)

Notes. The values are average from independent experiments (±SEM), P values indicate difference between treated with EMS and corresponding control samples (0 mM). * - significantly different.

The data demonstrate that the sensitivity of human whole blood leukocytes and isolated lymphocytes to the treatment with alkylating agent is approximately the same. Though, in this series of experiments, we have detected significant increase in the DNA content in the comet tail in leukocytes already at EMS concentration of 0.01 mM (p<0.012), while the significant level of DNA damage in lymphocytes was found only at higher concentrations of EMS (Table 6).

As a whole, the results demonstrate sufficient sensitivity of the comet assay in our modification for detection of damaging action of both ionizing radiation and alkylating agent on human whole blood leukocytes and isolated lymphocytes (Tables 5 and 6). In further experiments on studying genotoxic effects of HPPP EMF, we have used a treatment of cells with EMS in concentration of 5 mM as the positive control.

Taking into account the fact that under the influence of HPPP EMF the cell suspension will be overheated by 3.5±0.1°C, we studied the influence of different temperatures of incubation on occurrence of DNA damage in whole blood leukocytes and isolated lymphocytes. It was found that the incubation of the cells for 40 min at temperatures of 23, 27, 33, and 37°C does not significantly change a level of DNA damage in the cells (Table 7 and 9). Similarly, the exposure of the cells to HPPP EMF for 40 min does not induce any additional DNA damage compared to negative and sham-exposure controls (Tables 8 and 10). After exposure of the whole blood leukocytes to HPPP EMF, the percent of DNA in the comet tail was 1.26±0.26 that is not significantly different as compared to various temperature exposures (P>0.86). After exposure of the isolated lymphocytes to HPPP EMF, the percent of DNA in the comet tail was 0.38±0.05 that is not significantly different as compared to various temperature exposures (P>0.31). In contrast, the incubation of cells for 40 min at 37°C in the presence of EMS in concentration of 5 mM resulted in significant increase in DNA damage, the percent of DNA in the comet tail increased by more then 5 times comparing with corresponded control (Tables 8 and 10).

Table 7. Percent of DNA in the comet tail in human whole blood leukocytes incubated for 40 min at different temperatures.

Temperature of incubation	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 27°C)
23°C	1.07 ± 0.18	5	--	0.985
27°C	1.06 ± 0.21	6	0.985	--
33°C	1.13 ± 0.24	6	0.847	0.839
37°C	1.10 ± 0.20	8	0.921	0.917

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

Table 8. Percent of DNA in the comet tail in human whole blood leukocytes exposed to HPPP EMF for 40 min.

Exposure conditions	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 27°C)	P (compared to 37°C)
23°C	1.20 ± 0.20	18	--	0.746	0.890
HPPP EMF-exposure	1.26 ± 0.26	18	0.862	0.921	0.949
27°C	1.30 ± 0.18	11	0.746	--	0.833
37°C	1.24 ± 0.18	18	0.890	0.833	--
EMS 5 mM	6.77 ± 0.66	6	--	--	10^{-10}

Notes. The values are average from independent experiments (±SEM); P value indicate difference between various exposure conditions.

Table 9. Percent of DNA in the comet tail in human blood lymphocytes incubated for 40 min at different temperatures.

Temperature of incubation	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 27°C)	P (compared to 37°C)
23°C	0.55 ± 0.14	4	--	0.693	0.375
27°C	0.48 ± 0.04	3	0.693	--	0.540
33°C	0.41 ± 0.04	4	0.357	0.265	0.946
37°C	0.40 ± 0.09	5	0.375	0.540	--

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

Table 10. Percent of DNA in the comet tail in human blood lymphocytes exposed to HPPP EMF for 40 min.

Exposure conditions	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 37°C)
23°C	0.47 ± 0.06	10	--	0.784
HPPP EMF-exposure	0.38 ± 0.05	10	0.311	0.664
37°C	0.44 ± 0.12	8	0.784	--
EMS 5 mM	5.51 ± 0.74	8	--	10^{-5}

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

With the purpose of detection of possible DNA damage expressed some time later after the exposure to HPPP EMF, we have conducted a special series of experiments, in which after the 40-min exposure to HPPP EMF, whole blood leukocytes were additionally incubated for 30 min at 37°C. Before this series of experiments, we also studied the influence of different temperatures of incubation of the cells on occurrence of DNA damage. It was found that the additional incubation of blood samples for 30 min at 37°C leads to decrease in DNA damage, however, significant differences in percent of DNA in the comet tail in leukocytes preincubated at different temperatures are not manifested (Tables 7 and 11). The effect of HPPP EMF exposure was not also developed (Table 12). Percent of DNA in the comet tail decreased from 1.26±0.26 (without additional incubation) to 0.84±0.10 (with additional incubation), but insignificantly (P>0.3). This was also insignificantly different as compared to various temperatures of preincubation (P>0.6). In contrast, the additional incubation of the cells in the presence of EMS in concentration of 5 mM resulted in significant increase in DNA damage by more then 2 times (Table 12). Thus, the additional incubation of blood samples did not result in any development of damaging action of HPPP EMF. It could be explained by either the lack of direct damaging effects of HPPP EMF or high efficacy of repair systems removing DNA damage.

Table 11. Percent of DNA in the comet tail in human whole blood leukocytes preincubated for 40 min at different temperatures and then incubated for 30 min at 37°C.

Temperature of preincubation	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 27°C)	P (compared to without additional incubation at 37°C)
23°C	0.75 ± 0.26	5	--	0.919	0.335
27°C	0.78 ± 0.18	6	0.919	--	0.328
33°C	0.85 ± 0.18	5	0.744	0.775	0.395
37°C	0.73 ± 0.21	6	0.959	0.862	0.229

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

Table 12. Percent of DNA in the comet tail in human whole blood leukocytes exposed to HPPP EMF for 40 min and then incubated for 30 min at 37°C.

Exposure conditions	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 37°C)	P (compared to without additional incubation at 37°C)
23°C	0.94 ± 0.15	8	--	0.807	0.401
HPPP EMF-exposure	0.84 ± 0.10	8	0.618	0.824	0.312
37°C	0.88 ± 0.15	8	0.807	--	0.228
EMS 5 mM	16.0 ± 1.6	5	--	10^{-7}	0.0003

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

With the purpose of detection of possible DNA damage induced by HPPP EMF-exposure and expressed on the background of suppressed activity of repair systems, we have conducted a special series of experiments, in which after the 40-min exposure to HPPP EMF, whole blood leukocytes were additionally incubated for 30 min at 37°C in the presence of nonspecific inhibitor of repair sodium azide in concentration of 1 mM. It was revealed that this additional incubation of blood samples in the presence of sodium azide caused insignificant increase in DNA damage in leukocytes preincubated at 23°C (negative control) and exposed to HPPP EMF (Table 13), but significantly increased DNA damage in cells preincubated at 37°C without and with EMS (p<0.05) compared to without sodium azide data. The data analysis showed the lack of significant effect of HPPP EMF on whole blood leukocytes at additional incubation of the cells for 30 min at 37°C in the presence of nonspecific inhibitor of repair, comparing with negative controls (Table 13).

Table 13. Percent of DNA in the comet tail in human whole blood leukocytes exposed to HPPP EMF for 40 min and then incubated for 30 min at 37°C in the presence of sodium azide (1 mM).

Exposure conditions	Tail DNA ± SEM (%)	N	P (compared to 23°C)	P (compared to 37°C)	P (compared to without sodium azide data)
23°C	1.19 ± 0.13	6	--	0.086	0.243
HPPP EMF-exposure	1.24 ± 0.19	6	0.812	0.147	0.067
37°C	1.79 ± 0.31	5	0.086	--	0.049
EMS 5 mM	20.2 ± 1.0	6	--	10^{-7}	0.048

Notes. The values are average from independent experiments (±SEM); P values indicate difference between various exposure conditions.

Thus, on the basis of our results, we conclude that HPPP EMF under the chosen mode of exposure (8.8 GHz, 180 ns pulse width, peak power 65±5 kW, repetition rate 50 Hz, exposure duration of 40 min) does not cause any genotoxic effects on DNA of native human whole blood leukocytes and isolated lymphocytes *in vitro*. It could be explained by either the lack of direct damaging effects of HPPP EMF or high efficacy of repair systems, which have a time for removing of possibly induced by HPPP EMF-exposure DNA single-strand breaks and alkali-labile lesions detected by comet assay.
We have supposed that the additional incubation of cells at 37°C after termination of HPPP EMF-exposure would allow detection of any slowly repaired DNA damage which expressed some time later after the exposure. The analysis of results shows that the additional incubation of leukocytes for 30 min at 37°C after the termination of HPPP EMF-exposure did not exhibit significant DNA damage in native cells as compared to negative and sham controls.
With the purpose of detection of possible DNA damage induced by HPPP EMF-exposure and expressed on the background of suppressed activity of repair systems, we have conducted a special series of experiments, in which after the 40-min exposure to HPPP EMF, whole blood leukocytes were additionally incubated for 30 min at 37°C in the presence of nonspecific inhibitor of repair sodium azide in concentration of 1 mM. Sodium azide disengages the phosphorylation in mitochondria, suppresses ATP-dependent stages of repair processes, inhibits ATPase activity of series of enzymes [4, 5], in particular, DNA topoisomerase [13]. The results of these experiments showed the lack of significant effect of HPPP EMF on DNA in leukocytes at additional incubation of the cells in the presence of nonspecific inhibitor of repair.

4. Conclusions

The overall conclusion is that HPPP EMF under the chosen mode of exposure (8.8 GHz, 180 ns pulse width, peak power 65±5 kW, repetition rate 50 Hz, exposure duration of 40 min) does not cause any specific genotoxic effects on DNA of native frog erythrocytes, human whole blood leukocytes and isolated lymphocytes *in vitro*. The increase in DNA damage after exposure of frog erythrocytes to HPPP EMF was due to temperature rise in the cell suspension by 3.5±0.1°C, which can induce enhanced rate of apurinization/apyrimidinization of DNA at physiological conditions.

The present study leaves open a problem of HPPP EMF influence on activity of systems protected cells from damaging agents, that is very important at an assessment of combined influence of HPPP EMF and other environmental toxins. In this connection, an assessment of activity of repair systems under the influence of HPPP EMF has the particular concern. Let's

suggest that 40-min exposure of cells to HPPP EMF caused additional DNA damage. However, the repair system, actively operating in cells, does not allow accumulation of damaged DNA. For this reason, we and other investigators, studying dose dependencies of DNA damage under the action of ionizing radiation, conducted exposure at low temperatures, when the repair system is suppressed practically completely, that also results in increasing of the comet assay sensitivity. The experiments on exposure of cells to HPPP EMF at low temperature will require cooling a waveguide, that is enough complex technical problem requiring considerable efforts for modernization of equipment. The independent direction of further studies should be an assessment of genotoxic effects of HPPP EMF in experiments when the cells will be exposed in the presence of specific chemical agents inhibiting different stages of repair process. We could successfully accomplish the approaches noted here in spite of their laborious. The comparison of results obtained with the use of these approaches will allow one to answer the question whether HPPP EMF (8.8 GHz, 180 ns width, peak power 65 kW) can really cause genotoxic effects.

In pursuance of the work plan on the EOARD/ISTC #017011/#2350 project, we were based on a working hypothesis that HPPP EMF can cause genotoxic effects by direct DNA damage in cells, particularly, due to high electric field intensity in the exposed object. Now we offer a new working hypothesis, that HPPP EMF could cause genotoxic effects through the influence of HPPP EMF on aqueous phase of both cells and extracellular space. Our hypothesis is based on the following backgrounds. 1) The data exist on that the exposure of aqueous solutions to low-intensity centimeter or millimeter waves results in formation of reactive oxygen species (superoxide anion, peroxides, etc.) in these aqueous solutions [14, 15]. It is logical to suggest that the exposure of aqueous solutions to HPPP EMF in millimeter wave range could cause formation of reactive oxygen species in considerably higher concentrations. 2) It is known that the heightened generation of reactive oxygen species results in oxidizing damages of nucleic acids. Guanine is one of the most responsible targets for the influence of reactive oxygen species. As a result of the damage, 8-oxoguanine is produced, which is now considered as one of the basic biomarkers of oxidizing DNA damage. The relationship was revealed between formation of 8-oxoguanine and such processes as mutagenesis, carcinogenesis, aging and pathogenesis of elderly age diseases [9]. For careful verification of the proposed hypothesis, the solution of two high-priority tasks is necessary: 1) the quantitative measurement of reactive oxygen species forming in solutions under the exposure to HPPP EMF in millimeter wave range; 2) the quantitative assessment of biomarkers of oxidizing DNA damage, particularly, of 8-oxoguanine occurring in DNA of cells exposed to millimeter wave HPPP EMF. The quantitative assessment of 8-oxoguanine can be performed with the use of enzyme-linked immunosorbent assay.

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List of presentations at conferences and meetings

1. Chemeris N.K., Gapeyev A.B., Dudina M.V., Sirota N.P. Modification of technique for the analysis of nucleoid DNA structure on the basis of comet assay. In Proc. of International Conference "Genetic Consequences of Emergency Radiation Situations", Moscow, June 10-13, 2002, pp.306-308.

Abstract. Development and modification of special technique for the analysis of nucleoid DNA structure on the basis of the comet assay led to the following results. The method sensitivity was essentially increased due to application of special lysing conditions. The cost of the analysis was decreased owing to the use of visible light source for fluorescence excitation. The limitations of the method connected with high rate of preparation burning out at excitation of fluorescence of DNA-ethidium bromide complex by ultra violet light were abolished. The modifications produced were tested in model experiments and have shown their fitness.

2. Chemeris N.K., Gapeyev A.B., Sirota N.P., Skorochkina O.Yu., Konovalov I.V., Buzoverya M.E., Suvorov V.G., Logunov V.A. Assessment of the genotoxic effects of high peak-power pulsed electromagnetic field with the use of comet assay. In Proc. of Third International Symposium on Nonthermal Medical/Biological Treatments Using Electromagnetic Fields and Ionized Gases "ElectroMed 2003", San Antonio, Texas, June 11-13, 2003, pp.55-56.

Abstract. The purpose of the work was assessment of possible genotoxic effects of high peak-power pulsed electromagnetic field (HPPP EMF) on erythrocytes of frog Xenopus laevis and human blood lymphocytes. High-power square microwave pulses (8.8 GHz, 180 ns width, peak power up to 65±5 kW, repetition rate up to 50 Hz, average output power of 400±30 mW) were produced by a "Rodon" transmitter, made on the basis of a pulsed MI-522 magnetron. Cell suspensions were exposed in special plastic cuvettes of 80 μl in volume put inside a waveguide with a section of 23×3.4 мм^2. By means of measurements of temperature changes in the experimental cuvette filled with a normal saline solution, it was determined that an average initial rate of temperature rise was about 0.37±0.01^{o}C/sec, corresponding SAR was about 1.6 W/g (peak SAR was about 300 kW/g), and level of stationary overheating was about 3.5±0.1^{o}C. Cell suspension prepared for the exposure was divided into parts, the first of which was used for the negative control (a sample was placed into a not-energized section of the waveguide at room temperature of 21-23^{o}C), the second one was used for sham-exposure (a sample was placed into a not-energized section of waveguide at a temperature of 24-27^{o}C, which imitated the temperature rise induced by the exposure to HPPP EMF), the third one was used for the exposure, and the last one was used for the positive control (cells were incubated in the presence of ethyl methanesulfonate at concentration of 100 μM). Alkaline comet assay in our modification was used to assess DNA damage in cells. The fluorescence intensity of DNA-ethidium bromide complex was registered with the help of a modified light microscope, using a LED for fluorescence excitation. Standard parameters of "comets" were calculated on the basis of the analysis of 75-100 images of comets for each experimental point. Statistical analysis of the data was based on the Student t-test. Studying the influence of HPPP EMF on freshly isolated erythrocytes at exposure duration of 20 and 40 min, we have revealed only insignificant changes in percent of DNA in the comet tail, which were 0.4±0.4% and 0.7±0.7% decrease below the sham-control, respectively. Estimating the activity of DNA repair systems in nucleus, the exposure of erythrocytes to HPPP EMF was also conducted in the presence of 100 μM sodium azide, which is a nonspecific inhibitor of repair. At an exposure for 40 min, the percent of DNA in the comet tail was about 13.2±2.5% that was not significantly different from the sham-control (10.5±1.2%). Studying the influence of HPPP EMF on human blood lymphocytes at exposure duration of 40 min, we have revealed insignificant decrease in percent of DNA in the comet tail by 0.8±0.4% compared to the sham-control. In positive control, 40-min incubation of blood at 37^{o}C in the presence of 100 μM EMS, the percent of DNA in the comet tail was about 3.2±0.6% that was significantly larger (p<0.03) compared with corresponding control (incubation of blood at 37^{o}C), where the percent of DNA in the comet tail was about 1.7±0.2%. The results obtained allow us to conclude that HPPP EMF under the chosen mode of exposure did not cause any significant genotoxic effects on genetic material of frog erythrocytes and human blood lymphocytes in vitro. The work was funded by the grant from EOARD/ISTC #017011/#2350.

3. Gapeyev A.B., Chemeris N.K., Sirota N.P., Skorochkina O.Yu., Konovalov I.V., Buzoverya M.E., Suvorov V.G., Logunov V.A. High peak-power pulsed electromagnetic field does not cause any significant effect on chromatin of frog erythrocytes determined by the comet assay. In Proc. of 25th Annual Meeting of BEMS, Maui, Hawaii, June 22-27, 2003.

Abstract. OBJECTIVE: The purpose of the work was assessment of possible genotoxic effects of high peak-power pulsed electromagnetic field (HPPP EMF) on frog nuclear erythrocytes. METHODS: The work was performed with erythrocytes of frog Xenopus laevis. Cell suspension in concentration of 1×10^6 cells/ml was prepared in phosphate-buffered solution. High-power square microwave pulses (8.8 GHz, 180 nsec width, peak power up to 65±5 kW, repetition rate up to 50 Hz) were produced by a "Rodon" transmitter, made on the basis of a pulsed MI-522

magnetron. Cell suspension was exposed in special plastic cuvettes of cylindrical shape with a diameter of 10 mm and height of 1 mm (working volume of 80 µl) put inside a waveguide with a section of 23×3.4 мм^2 at a distance of 20-25 mm from waveguide flange. Using the microthermocouple, measurements of temperature changes in the experimental cuvette filled with a normal saline solution were carried out under the exposure to HPPP EMF at an average incident power of 400 mW. It was determined that an average initial rate of temperature rise was about 0.37±0.01^{o}C/sec, corresponding SAR was about 1.6 W/g (peak SAR was about 300 kW/g), and level of stationary overheating was about 3.5±0.1^{o}C. A suspension of erythrocytes was divided into three parts, the first of which was used for the negative control (a sample was placed into a not-energized section of the waveguide at room temperature of 20-22^{o}C), the second one was used for sham-exposure (a sample was placed into a not-energized section of waveguide at a temperature of 24-26^{o}C, which imitated the temperature rise induced by the exposure to HPPP EMF), and the third one was used for the exposure. To study changes in spatial organization of nucleoid DNA in erythrocytes, we have used the alkaline comet assay [1] in our modification [2]. The fluorescence intensity of DNA-ethidium bromide complex in "a comet head" was registered with the help of a modified light microscope, using a visible light lamp for fluorescence excitation. Average fluorescence intensity was calculated on the basis of the analysis of 75-100 images of comets for each experimental point. Statistical analysis of the data was based on the Student t-test. RESULTS: Three series of experiments were conducted. 1) Studying the influence of the temperature rise on chromatin spatial organization in erythrocytes, we did not found any significant effects of heating for 20-30 min in a temperature range from 25 up to 41^{o}C. 2) Studying the influence of HPPP EMF on freshly isolated erythrocytes at exposure duration of 20 and 40 min, we have revealed only insignificant changes in fluorescence intensity of DNA-ethidium bromide complex, which were 1.7±3.7% increase above and 5.9±3.6% decrease below the control, respectively. 3) Taking into account the existence of DNA repair in nucleus, the exposure to HPPP EMF was also conducted in the presence of 100 µM sodium azide, which is a nonspecific inhibitor of repair. At an exposure for 40 min, the decrease in fluorescence intensity was about 18±6% that was not significantly different from the corresponding control (imitation of the temperature rise induced by the exposure - 17±6%). CONCLUSION: The results obtained allow us to conclude that HPPP EMF under the chosen mode of exposure (8.8 GHz, 180 nsec width, peak power up to 65±5 kW, repetition rate of 50 Hz, exposure duration 20 and 40 min) did not cause any significant genotoxic effects on frog erythrocytes in vitro. References: 1. N.P. Sirota, V.G. Bezlepkin, E.A. Kuznetsova, M.G. Lomayeva, I.N. Milonova, V.K. Ravin, A.I. Gaziev, R.J. Bradbury. Modifying effect in vivo of interferon alpha on induction and repair of lesions of DNA of lymphoid cells of gamma-irradiated mice. *Radiat. Res.* 1996, 146 (1), 100-105. 2. N.K. Chemeris, A.B. Gapeyev, M.V. Dudina, N.P. Sirota. Modification of technique for the analysis of nucleoid DNA structure on the basis of comet assay, in: Proc. of International Conference "Genetic Consequences of Emergency Radiation Situations", Moscow, June 10-13, 2002, p.306-308. The work was funded by the grant from EOARD/ISTC #017011/#2350.

Project Manager	Director of the Institute of Cell Biophysics RAS
Nikolai K. Chemeris	Evgeny E. Fesenko
Date: 20.06.2003	Date: 20.06.2003

PROJECT SUMMARY FOR UNRESTRICTED DISTRIBUTION

Summary of the project

The objective of this project is the detection of possible genotoxic effects of high peak-power pulsed electromagnetic fields (HPPP EMF). The research is a basic research. The experiments were performed with nuclear erythrocytes of *Xenopus laevis* frog, human whole blood leukocytes and isolated lymphocytes obtained from fresh donor blood commercially purchased at a blood transfusion station. The basic experimental method was the alkaline comet assay (single cell gel electrophoresis assay) with several modifications. The comet assay is high sensitive method to detect DNA single-strand breaks and alkali-labile lesions caused by the influence of different damaging agents. As a result of modifications of the equipment used for image analysis, a mobile variant of photometry setup with total weight of about 3 kg was established. The slides were examined under a modified light microscope with luminescent header, using a super bright LED for fluorescence excitation. CCD camera with a high sensitivity and resolution was used for recording the images of nucleoids. The software created for recording and online image analysis has permitted to achieve a rate of image analysis up to 400 comets per hour. Algorithms for image filtering and smoothing, for calculation of standard parameters of "comets" (amount and percent of DNA in the comet head and tail, radius of the comet head, tail length, tail moment, Olive tail moment and integral fluorescence intensity in the comet head by a fixed threshold) were realized. Modification of the equipment and software, application of digital methods for registration and image analysis have allowed the essential optimisation of the method, increasing the rapidity and decreasing the cost price of the analysis.

Because of the high prices of equipment for the exposure of biological samples to HPPP EMF, all HPPP EMF exposures and parallel controls were conducted under subcontract at the Russian Federal Nuclear Center - All-Russian Research Institute of Experimental Physics (Sarov, Novgorod Region), possessing the appropriate equipment. High-power square microwave pulses (8.8 GHz, 180 ns width, peak power up to 65±5 kW, repetition rate up to 50 Hz, average incident power of 400±10 mW) were produced by a "Rodon" transmitter, made on the basis of a pulsed MI-522 magnetron. Cell suspension was exposed in special plastic cuvettes of cylindrical shape with a diameter of 10 mm and height of 1 mm put inside a waveguide with a section of 23×3.4 мм^2 at a distance of 25-30 mm from waveguide flange. From the temperature kinetics it was determined that for the 50 µl of physiological saline, an average initial rate of temperature rise was about 0.37±0.01^{o}C/s, corresponding SAR was about 1.6 kW/kg (peak SAR was about 300 MW/kg), and the level of stationary overheating was about 3.5±0.1^{o}C. Blood samples and lymphocyte suspension were exposed to HPPP EMF in parallel with sham-exposure, negative and positive controls.

The sensitivity of isolated erythrocytes of *X. laevis* to action of different doses of γ-ray ionizing radiation (0, 50, 100, and 200 cGy) and directly influencing DNA alkylating agent ethylmethane sulfonate (EMS) in concentration of 5 mM was assessed. The extended analysis of the data has shown that the demonstrative quality and quantitative representation of DNA damage in the cells and least variability are given by averaged data on percent of DNA in the comet tail. The dose of 50 cGy significantly increased the DNA content in the comet tail by more than 3 times as compared to the corresponding control (0 cGy). EMS in concentration of 5 mM during 40-min incubation at 20^{o}C increased the DNA content in the comet tail by approximately 4 times as compared to the corresponding control (0 mM). The data obtained show that in spite of highly effective system of DNA repair in frog, the comet assay detects significant DNA damage in frog erythrocytes in vitro exposed to ionizing radiation and 5 mM EMS.

The sensitivity of human whole blood leukocytes and isolated lymphocytes to the action of different doses of X-ray ionizing radiation (0, 150, 274, and 548 cGy) and alkylating agent EMS was studied. It was shown that occurrence of DNA damage in the cells depends pronouncedly on the dose of ionizing radiation. The X-ray radiation at a dose of 274 cGy increased the DNA content in the comet tail in leukocytes by 2.7 times ($p<0.001$) and in isolated lymphocytes by more than 6 times ($p<0.001$). After 20-min incubation of the cells with different concentrations of EMS (from 0.001 to 10 mM) at 37±0.5^{o}C, significant increase in the DNA content in the comet tail in leukocytes ($p<0.012$) was detected already at EMS concentration of 0.01 mM, while the significant level of DNA damage in lymphocytes was found only at higher concentrations of EMS.

As a whole, the results demonstrate high sensitivity of the comet assay for detection of damaging action of both ionizing radiation and alkylating agent on frog erythrocytes, human whole blood leukocytes and isolated lymphocytes. In experiments on studying genotoxic effects of HPPP EMF, a treatment of cells with EMS in concentration of 5 mM was used as the positive control.

The influence of different temperatures (20, 25, and 30^{o}C) of incubation of cell suspension on DNA integrity in frog erythrocytes was studied. The results indicated the increase in DNA damage in erythrocytes with temperature elevation. The DNA damage caused by incubation of cell suspension at 30^{o}C for 40 min was significantly ($p<0.003$ and $p<0.02$) higher as compared to 20^{o}C and 25^{o}C temperature conditions, respectively. Considering the DNA damage due to temperature elevation during 40-min exposure of cells to HPPP EMF, the data on incubation of cell suspension at 30^{o}C, sham- and HPPP EMF exposure were collated. The averaged data demonstrate the same percent of DNA in the comet tail in sham-exposed and HPPP EMF exposed erythrocytes ($P>0.99$).

Taking into account the overheating of cell suspension by 3.5±0.1^{o}C under the influence of HPPP EMF, the influence of different temperatures of incubation on occurrence of DNA damage in whole blood leukocytes and isolated lymphocytes was studied. It was found that the incubation of the cells for 40 min at temperatures of 23, 27, 33, and 37^{o}C does not significantly change a level of DNA damage in the cells. Similarly, the 40-min exposure of the cells to HPPP EMF did not induce any additional DNA damage compared to negative and sham-exposure controls. After exposure of the whole blood leukocytes and isolated lymphocytes to HPPP EMF, the percent of DNA in the comet tail was 1.26±0.26 and 0.38±0.05, respectively, that was not significantly different as compared to various temperature exposures ($P>0.86$) and ($P>0.31$). The additional 30-min incubation of blood samples at 37^{o}C after termination of HPPP EMF-exposure did not result in any development of damaging action of HPPP EMF ($P>0.6$). Either the lack of direct damaging effects of HPPP EMF or high efficacy of repair systems removing DNA damage could explain it. After 40-min of exposure to HPPP EMF, whole blood leukocytes were additionally incubated at 37^{o}C for 30 min in the presence of 1 mM sodium azide nonspecific inhibitor of repair. The results showed the lack of significant effect of HPPP EMF on DNA in leukocytes at additional incubation of the cells in the presence of nonspecific inhibitor of repair.

The overall conclusion is that, under the chosen mode of exposure (8.8 GHz, 180 ns pulse width, peak power 65±5 kW, repetition rate 50 Hz, exposure duration of 40 min), HPPP EMF does not cause any specific genotoxic effects on DNA of native frog erythrocytes, human whole blood leukocytes and isolated lymphocytes *in vitro*. The increase in DNA damage after exposure of frog erythrocytes to HPPP EMF was due to temperature rise in the cell suspension by 3.5±0.1^{o}C.

The present study leaves open a problem of HPPP EMF influence on activity of systems protected cells from damaging agents, that is very important at an assessment of combined influence of HPPP EMF and other environmental toxins. In this connection, an assessment of

activity of repair systems under the influence of HPPP EMF will have the particular concern in further studies.

List of publications

1. Chemeris N.K., Gapeyev A.B., Dudina M.V., Sirota N.P. Modification of technique for the analysis of nucleoid DNA structure on the basis of comet assay. In Proc. of International Conference "Genetic Consequences of Emergency Radiation Situations", Moscow, June 10-13, 2002, pp.306-308.

2. Chemeris N.K., Gapeyev A.B., Sirota N.P., Skorochkina O.Yu., Konovalov I.V., Buzoverya M.E., Suvorov V.G., Logunov V.A. Assessment of the genotoxic effects of high peak-power pulsed electromagnetic field with the use of comet assay. In Proc. of Third International Symposium on Nonthermal Medical/Biological Treatments Using Electromagnetic Fields and Ionized Gases "ElectroMed 2003", San Antonio, Texas, June 11-13, 2003, pp.55-56.

3. Gapeyev A.B., Chemeris N.K., Sirota N.P., Skorochkina O.Yu., Konovalov I.V., Buzoverya M.E., Suvorov V.G., Logunov V.A. High peak-power pulsed electromagnetic field does not cause any significant effect on chromatin of frog erythrocytes determined by the comet assay. In Proc. of 25th Annual Meeting of BEMS, Maui, Hawaii, June 22-27, 2003.

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